OBSERVATIONS ON THE GROWTH OF STREPTOCOCCI IN BLOOD-CARBOHYDRATE MEDIUM *

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Carbohydrates not only serve as an important food substance for many bacteria but are most useful for the identification and differentiation of closely related varieties. Blood and blood serum in recent years have come into use quite extensively as an aid in differentiation of organisms. In addition to other means the ability of germs to lake the corpuscles has been taken advantage of, especially in connection with the examination of cocci. The interaction, therefore, of blood and carbohydrates in mediums under the influence of bacterial growth is of significance both practically and theoretically.

Since Schottmüller called attention to the hemolytic action of streptococci on blood and on this basis divided them into 3 types: the hemolyticus, the viridans, and the mucosus, this method has in many laboratories come into general use for isolating and differentiating this group. For this purpose it has proved an invaluable aid. By many workers, especially in this country for the past 15 years, the method has been tested out quite thoroughly and its limitations and possibilities have been fairly well determined. I may refer in this regard to the early work in this country of Weaver, Ruediger, and Rosenow, to the more recent work of Smith and Brown,[1] and to the classifications of streptococci advocated by Holman[2] and by Lyell;[3] also to the recent work of Becker.[4] The hemolytic property, like many other properties of bacteria, is not absolutely constant but ordinarily under uniform conditions is sufficiently so to be of real value in differentiation.

It has long been known that sugar in mediums interfered with the production of characteristic zones of hemolysis on plate cultures.

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1 Jour. Med. Research, 1914, 31, p. 455.
2 Ibid., 1916, 34, p. 377.
3 Ibid., 1914, 30, p. 487.
4 Jour. Infect. Dis., 1916, 19, p. 754.

It was pointed out by Ruediger[5] that in glucose blood mediums hemolytic streptococci failed to produce a clear zone, but became surrounded by an indefinite greenish or brownish zone. He showed also that lactic acid when dropped on a blood-agar plate caused a similar greenish zone to form about itself. Cole[6] has demonstrated that pneumococci change oxyhemoglobin to methhemoglobin in artificial medium, and that the greenish or greenish-brown color about pneumococcus colonies on blood plates is due to this phenomenon. Blake[7] has made the same observation with streptococcus viridans. Sugars very appreciably accelerate this phenomenon.

Hiss[8] called attention to the value of inulin serum water mediums in the differentiation of pneumococci and streptococci. As a result of the fermentation of inulin by pneumococci, there resulted from the acids formed a precipitation and coagulation of the albuminous substances in the medium, causing it to become white and solid. This change does not occur in such medium inoculated with streptococci. This method has been modified by Ruediger,[9] who used a higher proportion of serum or ascites fluid in order to facilitate the growth of certain strains of pneumococci, which did not grow well in Hiss serum water medium.

Blood mediums should be standardized more carefully than they have been in the past. Attention has been called to this by nearly everyone who has worked on the subject. Uniform methods should be used so that the results of various workers may be comparable.

Blood, when used in mediums, should be reasonably fresh. Standing in a cool chamber for several days, however, does not impair its value to any marked degree. There are certain appreciable differences between human blood and the blood of various animals as shown by Becker.[4] One-half c.c. of defibrinated human blood added to 5 c.c. of melted agar cooled to approximately 42 C. is the proportion of blood used in the experiments herein reported.

With a series of strains of streptococci, I have made observations on the effect of their growth on blood mediums containing a variety of sugars both fermentable and nonfermentable; 1% sugar mediums were first used, but on account of an occasional indefinite or delayed reaction the amount of sugar was increased to 2 or 3% in the mediums and either one of these amounts was used in the later experiments. I should recommend 2% for routine work.

It was noted that when streptococci are grown on fermentable sugar blood mediums, the mediums acquire a brown turbidity, beginning at first near the colonies, and later involving the entire plate or tube. This change may be attributed to the action of acids produced

[5] Jour. Infect. Dis., 1906, 3, p. 663.
[6] Jour. Exper. Med., 1914, 20, p. 363.
[7] Ibid., 1916, 24, p. 315.
[8] Ibid., 1902, 10, p. 317.
[9] Jour. Am. Med. Assn., 1906, 47, p. 1171.

by the bacteria, and in order to analyze this point further systematic tests have been made of the affects of the growth of a number of strains of streptococci on mediums containing the various sugars. The results are given in Table 1.

This table shows the fermentation of sugars as determined by titration, and it also indicates by the plus sign those blood-sugar plates and tubes which developed the striking brown turbidity. It will be seen that the reaction as manifested by the development of brown turbidity corresponds in every instance to the positive fermentation tests. With those sugars which are not fermented by the streptococci the blood mediums remain red, and the typical clear zones of hemolysis on plate cultures are quite like those produced on plain blood-agar plates.

TABLE 1

EFFECT OF STREPTOCOCCI WHEN GROWN ON CARBOHYDRATE (2%) BLOOD AGAR PLATES AND TUBES

Number of Strain	Glucose		Lactose		Maltose		Salicin	
	Blood Reaction	Titration	Blood Reaction	Titration	Blood Reaction	Titration	Blood Reaction	Titration
72	+	6.05	+	4.37	+	5.05	+	6.05
183	+	5.15	+	5.05	+	5.05	+	5.05
300	+	6.25	+	4.55	+	5.55	+	5.05
228	+	6.35	+	2.71	+	5.25	+	4.71
140	+	5.87	+	4.55	+	5.05	+	4.85
290	+	5.05	+	4.65	+	3.35	+	5.05
41	+	6.55	+	3.65	+	5.25	+	4.05
134	+	6.37	+	4.55	+	4.05	+	4.55
310	+	6.05	+	4.87	+	5.05	+	5.35
6	+	6.10	+	4.60	+	5.30	+	5.10
211	+	4.55	+	3.65	+	3.85	+	4.35

These reactions are so clear cut that sugar blood tubes may be used for determining fermentation properties instead of litmus mediums. By adding a small amount of lactic acid to mediums, either in plate or tube, the change that occurs is quite indistinguishable from that caused by the growth of the acid-producing bacteria.

The zone of hemolysis on the fermentable sugar-blood plates was often indistinct, and at times not visible at all. Sometimes the zone at first was fairly clear, later becoming more turbid, but not persisting as a clear and definite zone, like that which hemolytic streptococci produce on the plain blood mediums.

This reaction is entirely comparable to the reaction observed in the sheep serum inulin mediums devised by Hiss for the differentiation of streptococci and pneumococci. When the defibrinated blood is used we get not only a precipitation of the serum constituents, but also a brownish discoloration of the red blood cells, due to acids and no doubt other changes which make the differentiation decidedly

striking. Since blood and carbohydrates are no doubt coming more and more into use in growing and determining bacteria, I suggest this method as of importance in the study and classification of members of the pneumo-streptococcus group.

An attempt was made to test the relation of hemolysis to acid-production by streptococci. An experiment was designed to neutralize the effect of the acid by the addition of powdered $CaCO_3$ to the blood-sugar mediums.

Glucose and lactose were used. On such mediums when freshly made and plated the particles of $CaCO_3$ are seen evenly distributed. After incubation the $CaCO_3$ particles around the streptococcus colonies are dissolved, to some extent in 24 hours, much more so in 48 or more hours. The zone of solution of $CaCO_3$ particles is larger than the zone of hemolysis. For 24-48 hours

TABLE 1.—*Continued*

EFFECT OF STREPTOCOCCI WHEN GROWN ON CARBOHYDRATE (2%) BLOOD AGAR PLATES AND TUBES

Saccharose		Mannite		Raffinose		Inulin	
Blood Reaction	Titration	Blood Reaction	Titration	Blood Reaction	Titration	Blood Reaction	Titration
+	Acid	+	3.35	0	1	0	0.95
+	Acid	0	1.00	0	1	0	0.95
0	1.5	0	1.00	0	1.1	0	1.25
+	4.65	0	1.5	0	1.5	0	1
+	3.55	+	3.05	0	1	0	0.91
+	5.05	0	0.95	0	0.83	0	1.2
+	4.25	0	0.95	0	1	0	1
+	4.6	0	0.98	0	0.85	0	1.08
+	5.55	0	0.9	0	1.2	0	0.91
+	4.70	0	1	0	0.9	0	1.08
+	3.55	0	0.9	0	1	0	1.08

the blood on the $CaCO_3$ plates does not change materially; the carbonate seems to be appreciably protective for the corpuscles for a time at least. Later the plates slowly turn brownish and turbid, but usually not to the same extent as in the plates without $CaCO_3$. The colonies on the $CaCO_3$ plates become somewhat larger than when grown on mediums without $CaCO_3$.

Control experiments were made to test the effect of $CaCO_3$ on plain blood-agar plates and the streptococcus hemolytic zones. The plain blood agar containing $CaCO_3$ remains red and appears fresh for a week or more, at room or incubator temperature, and shows no difference from a control plate made without $CaCO_3$. On $CaCO_3$ blood plain agar plates sown with hemolytic streptococci, the zones are about normal in size and appearance and remain so for a week or longer. About the colonies on such plates the particles of $CaCO_3$ remain indefinitely. Therefore the zone of hemolysis is not affected by $CaCO_3$, and it would seem further that it bears no relation to acid-production in the case of typical hemolytic streptococci. Twenty strains were tested and all reacted alike.

The question as to whether sugars inhibit hemolysis was inquired into, using the blood-agar plate. Six typical hemolytic streptococci were tested first on plain blood plates to determine the diameter of the zone of hemolysis. This was found to vary 2-4 mm. for the various strains. They were also

plated on $CaCO_3$ blood-glucose agar, and the size of the zones were noted and compared with the controls. The average size of the zones of many colonies of each of the strains revealed no essential differences. It was noted here again that the $CaCO_3$ particles in the colony, and for some distance about it, were dissolved in the sugar mediums. The clearing zone of the $CaCO_3$ particles is decidedly larger than that of the blood corpuscles. This is another point indicating that the zone of hemolysis is not dependent on acid-production. That acid- and hemolysin-production go on together was shown by these experiments, but it was noted that apparently the rate of the acid-production is relatively slower than that of hemolysis. At any rate the blood corpuscles seem more sensitive to hemolysin than do the particles of $CaCO_3$ to the acid in the concentration in which these substances exist about the colonies.

It would appear that the acid is decidedly more diffusible in the mediums than is the hemolysin. The hemolysin diffuses into the medium rather rapidly during the first 24 hours, then slowly as judged from the size of the zone. Furthermore, the margin, in most strains of streptococci but not in all, is very clear cut and definite. On the sugar plates the acid diffuses rather rapidly, and continues to diffuse for several days so that, as a rule, sooner or later the entire plate becomes turbid and brown, even though few colonies may be found on it. The margin of the acid zone also is not clear and definite as is usually the margin of the zone of hemolysis.

Since mediums are commonly made with meat or extract which contains some muscle sugar, the point whether or not such sugar has any appreciable effect on plate hemolysis was inquired into. Eight typical hemolyzing strains of streptococci were selected for the tests Plain agar was prepared, using meat; also the same medium, using ordinary meat extract. As controls sugar-free agar was used. On all these mediums no differences were noted between the character of the hemolytic zones about the streptococci. It would seem that the small amount of sugar in such mediums is too small to appreciably affect the nature or intensity of hemolysis.

Attention is called to the fact that certain peptones, on the market especially since the war, contain considerable quantities of sugar, and when such are used in the preparation of mediums, they are not suitable for the differentiation of bacteria by blood-agar methods. When Witte's peptone is not available the peptone used should be investigated as to its sugar content. This point is especially important in the preparation of standard medium and particularly of sugar-free medium. I would point out also that certain peptones darken the medium to such an extent that clear cut blood reactions are not readily obtained.

The amount of NaCl in medium is important in relation to hemolysis. When no salt is added, as is well known, blood introduced into such mediums immediately hemolyzes. Plain agar was prepared containing amounts of NaCl, 0.1%-1.5%. Ten strains of hemolytic streptococci, 5 virulent and 5 nonvirulent, were tested on this medium to which one-third c.c. of human defibrinated blood to 5 c.c. was added before plating. The fragility of the corpuscles in this medium is a little below the resistance in pure salt solution, as might be expected. At 0.4%, at which in pure salt solution there is slight hemolysis, there was apparently no hemolysis as observed both with the microscope and the naked eye. Above this point the zones of hemolysis were normal in the various concentrations tested. If to such salft-free medium or medium with low NaCl content, sugar, either fermentable or nonfermentable, be added in the usual concentration of 1% or in any sufficient concentration, hemolysis will not take place, because the sugar protects the corpuscles. For such medium, therefore, salt is not a necessary constituent for the prevention of hemolysis.

SUMMARY

Streptococci when grown on a fermentable sugar-blood medium cause a characteristic brownish turbidity which soon involves the entire tube or plate.

On nonfermentable sugar-blood medium no such change is noticed, hemolysis occurring as on plain blood mediums.

This change is sufficiently definite and constant to be of value in determining carbohydrate reactions.

It is useful in routine work since practically all varieties of streptococci grow well in this medium.

Powdered $CaCO_3$ added to sugar-blood agar will protect the medium for a time against the acids formed, but not permanently.

The carbonate added to plain blood medium has no appreciable effect on hemolysis by streptococci.

The presence of sugar in various peptones, at present on the market, is a disturbing factor in the blood-plate culture of bacteria.